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New record of *Chaetomium bostrychodes* Zopf and *Neoscytalidium novaehollandiae* Pavlic. as wheat seed-borne mycoflora (*Triticum aestivum* L.) in Iraq

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The present study aimed to identify fungal species, specifically Chaetomium bostrychodes and Neoscytalidium novaehollandiae, isolated from seeds of five wheat cultivars: Mahmoudia (MHD), Babil (BBL), Bohooth (BTH), Adena (ADN), and Wefia (WAF). Phenotypic identification of both fungi was conducted based on morphological and microscopic characteristics. Molecular diagnosis, employing internal transcribed spacer primers (ITS1 and ITS4), was utilized to ascertain the species' identity. The morphometric and microscopic results for both fungi demonstrated a consistent agreement with previously published findings related to fungal features. Molecular sequences further confirmed the identity of these species, with a sequence similarity percentage of 90% for C. bostrychodes (KP216865.1 deposited at https://www.ncbi.nlm.nih.gov/genbank/) and 98% for N. novaehollandiae (MT397136.1). Notably, this study marks the first documentation of C. bostrychodes and N. novaehollandiae as wheat seed-borne fungi in Iraq. The molecular confirmation adds a robust layer to the identification process, providing a comprehensive understanding of the fungal species present in the examined wheat cultivars. This novel report contributes valuable insights into the fungal diversity associated with wheat seeds in the region.

Keywords: *Chaetomium bostrychodes*, morphology, *Neoscytalidium novaehollandiae*, Phylogenetic analysis, wheat seeds, Fungal species, Chaetomium bostrychodes, wheat cultivars, Morphometric analysis.

INTRODUCTION

Wheat *Triticum aestivum* L. belongs to the family Poaceae (previously known Gramineae) (Abass *et al*., 2021). Wheat is the main grain crop supporting both global agriculture and the economy in the world. Additionally, it is an important crop grown for production of grains providing food sources for humans and livestock animals (Kochhar, 2016; AL-Abody *et al*., 2021). Grote *et al*. (2021) stated that wheat is the main grown grain crop occupying 17% of the entirely agrarian lands in the world (Grote *et al*., 2021). The European Union, Australia, the United States, Pakistan, Turkey, Canada, Russia, China, India, Turkey and Ukraine are the most nations growing wheat with up to 80% of international production (Shad *et al*., 2019). According to FAOSTAT (2023), more than 220 million hectares were globally cultivated with wheat producing approximately 770 million tonnes in 2021. In Iraq, it is grown in winter season considering the most essential crop consumed locally (Ewaid *et al*., 2020). CSO (2023) confirmed that more than 1.8 million hectares was cultivated with this crop, and its production was approximately 2.7 million tonnes.

Although the wheat crop supports global food security, it suffers from pre-harvest and postharvest damage in the growth of its plants and their productivity (Ulziijargal *et al*., 2019). The activity of seed-borne fungi is one of the most destructive causes of the quality and quantity of wheat seeds; particularly those fungi that cause disease on crop plants in the fields and move with seed yield into granaries (Khan *et al*., 2023). Additionally, Shad *et al*. (2019) showed that the infected seeds become less resistant to the attack of other fungal species in the stores. This leads to more contamination with various fungal pathogens and more reduction in the value of seeds during the period of storage (Ilyas and Manohara, 2023). Seed-borne pathogens that either exist internally or externally or associate with wheat seeds as contaminants are able to cause seed decay, seed necrosis, decrease of germination vigor, and seedling damping-off, all of which lead to progress of disease during phases of crop growth through local or systemic infection. Infected seeds are

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considered the most effective carriers of phytopathogens for far distances (Dhakar *et al*., 2018).

With regard to *C. bostrychodes*, it is saprophytic fungus belongs to Phylum: Ascomycota, Class: Sordariomycetes; Order: Sordariales; Family: Chaetomiaceae and genus *Chaetomium* (Kubatova, 2006). The genus includes more than 390 species that have been globally reported and only 273 were approved in accordance with the Index Fungorum Partnership (IFP) (Abdel-Azeem, 2020). Species of the genus commonly present in varied habitats such as seeds, soil, remains of rot plants and others (Abdullah and Azzo, 2015). Asgari and Zare (2011) mentioned different species of the genus *Chaetomium* as contaminants isolated from different sources, including wheat, barley, citrus, stored cotton, and diagnosed five species from seeds of wheat crop basing on morphological and molecular features. Abdullah and Azzo (2015) also reported some species of this genus, including *C. bostrychodes*, isolated from soil of grapevine in Iraq. According to Abdel-Azeem (2020), several species of the genus *Chaetomium* synthesize mycotoxins, such as sterigmatocystin, mollicellins, chaetochromin and chaetoglobosins.

On the subject of *N. novaehollandiae*, it has been increasingly recorded as a phytopathogen considered one of the most important causal agents of disease on wide range of plant genera in recent years (Alizadeh *et al*. 2022). The fungus was classified as a member of the Phylum: Ascomycota, Class: Dothideomycetes; Order: Botryosphaeriales; Family: Botryosphaeriaceae and genus *Neoscytalidium* (Bakhshi *et al*., 2022). Species of the genus can potentially infect different plants causing diseases which exhibit various symptoms on underground and aerial parts of plant hosts. Those symptoms include root rot and black canker, collar root rot, needle and shoot blight, dry and black root rot, defoliation, tuber rot, interior stem necrosis, elongated canker, fruit and stem canker, fruit rot and shoot blight, shoot blight, brown spot, internal brown rot of fruit, canker, leaf blight, stem rot and plant death (Alizadeh *et al*. 2022). Ray *et al*. (2010) first identified *N. novaehollandiae* and other species as fungal pathogens causing dieback disease on *Mangifera indica* tree in Australia. In their study, Alizadeh *et al*. (2022) also confirmed that the fungus was a causal agent of dieback disease on *Pinus eldarica* trees and the authors described its morphological and molecular characteristics. Moreover, Abdulrahman and Haleem (2023) reported that *N. novaehollandiae* causes dieback and canker diseases on *E*ucalyptus *microtheca*, *E. calmaldulensis* trees and *Melia azedarach* trees in Iraq. Furthermore, Abdul-Karim and Aljarah, (2021) identified the fungus morphologically and molecularly as phytopathogen causing branches wilt and sooty stem diseases on pomegranate (*Punica granatum*), apple (*Malus domestica*), mulberry (*Morus alba*), castor (*Ricinus communis*) and india rubber (*Ficus elastica*) trees in Iraq. Dervis *et al*. (2020) first reported *N. novaehollandiae* as causal agent of stem blight disease on tomato plants in Turkey. Because of locally limited bio-data sources of both fungi on wheat, the aim of the current study is to focus particularly on identifying exact *Chaetomium bostrychodes* and *Neoscytalidium novaehollandiae* as seed-borne fungi on wheat seeds for the first time in Iraq.

MATERIALS AND METHODS

***Experimental materials*:** In December 2022, seed samples of five wheat genotypes were collected randomly from farmers' stores and local markets. Those samples were kept in sterile plastic bags and then shifted in refrigerator at 5° C until use. There were three types of artificial media; Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) and Water Agar (WA) were used in culturing stage. The reagents were Favorgen Biotech Corp Kit for Extraction of rapid fungi genomic DNA and the application of PCR. The primers were Internal Transcribed Spacer ITS1 and ITS4.

The lab tools were optical microscopes (dissecting and light microscopes (Olympos/ Germany), Laminar flew cabinet, autoclave, constant temperature bath, centrifuge, instrument of Polymerase Chain Reaction PCR, gel electrophoresis equipment, constant temperature and others.

EXPERIMENTAL METHODS

***Isolation and purification of fungi*:** Primarily, 5% sodium hypochlorite was used to sterilize seed samples for three minutes. This was followed by washing those seeds with sterile distilled water for three times, and then dried on sterilized filter papers. Next, eight of sterilized seeds were selected randomly and plated on 90 mm PDA Petri dish. For each wheat cultivar, there were three PDA and MEA plates of sterile seeds were prepared as replicators. Those dishes were moved into the incubator set at 25° C ±1 within the darkness condition for nine days. The entire grown fungi were isolated from each initial fungal culture and then purified on water agar (WA) medium. The pure isolates were maintained on quarter-strength PDA and sterile 20 mL vials of sterilized PDA and MEA slant media. The isolation and purification processes were performed under standardized conditions.

***Detection of fungi*:** The primary detection of examined fungal species was carried out using a dissecting microscope to observe the morphological characteristics of fungi including vegetative and sexual reproductive structures. While, the morphology of pure colonies was noticed within the period of fungal growth, light microscope (Olympos/ Germany) was utilized after preparing glass slides to identify microscopic features of two investigated fungi. The isolates of fungi were identified to correspond with those mentioned in related studies of *C. bostrychodes* (Kubatova, 2006; Asgari and Zare, 2011; Abdel-Azeem, 2020; Al-Dossary *et al*., 2021) and *N. novaehollandiae* (Slippers *et al*., 2009; Sabernasab *et*

Table 1. Sequence of ITS primers applied in PCR amplification.

No.	Primer	Sequence	Length	Reference	Supplier
1	ITS1 F	5-TCCGTAGGTGAACCTGCGG-3	19	Ahmed and Abass	Bioneer
2	ITS4 R	5-TCCTCCGCTTATTGATATGC-3	20	(2022)	

Table 2. PCR Conditions utilizing ITS universal primer.

No.	Step	Temperature / ºC	Time	Cycles No
1	Denaturation 1	94	5 min.	1
2	Denaturation 2	94	30 sec.	25
3	Annealing	56	45 sec.	25
4	Extension	72	1 min.	25
5	Final Extension	72	7 min.	1

al., 2019; Abdul-Karim and Aljarah, 2021; Alizadeh *et al*., 2022; Abdulrahman and Haleem, 2023).

***Molecular diagnosis and phylogenetic analysis of fungal isolates*:**It was performed by using the International Molecular Code for ITS1 and ITS4 by PCR to amplify the pieces of genome DNA according method of Abass (2017). Moreover, the reagent of PCR analysis was applied in reaction of PCR including 5 µL of master mix, 1 µL of forward ITS1, 1 µL of reverse ITS4, 13 µL nuclease free water (dd H_2O) and 5 µL of DNA template. The total volume was 25 µL used in experiment. Additionally, the Universal Primers ITS1 and ITS4 for amplification process were shown in Table 1. Regarding to the PCR reaction, it was carried out at volume of 25 µl (Table 2).

***Phylogenetic Analysis of Fungal Species*:** The NCBI / BLAST (National Centre of Biotechnology: https://www.ncbi.nlm.nih.gov/ and Basic Local Alignment Search Tool: https://blast.ncbi.nlm.nih.gov/Blast.cgi) were followed to search the nucleotide sequences for each examined fungal species and comparing their sequence with identified species. The sequence of each fungal species gene was deposited at NCBI as advised. The phylogenetic trees were drawn for the fungal species using MEGA-X software 11 by Maximum Likelihood method (Jones et al., 1992; Tamura et al., 2021; Ahmed and Abass, 2022).

RESULTS AND DISCUSSION

***Morphological and microscopic identification of the fungi*:** The main features of *C. bostrychodes* observed were that colonies were creamy and grew slowly reaching 44 mm in diameter in 12 days at 24° C under dark incubating conditions. Mycelia were mostly flooded, grey to dark brown. Chlamydospores were pale yellow, sub-globose to globose 10-12 µm in width. Ascomata were deep brown to black, superficial, diffused, and sub-globose to globose measured 285-325 µm in length 275-305 µm in width. Terminal hairs were smooth, in bundle originated surrounding the ostiole, separated at the top measured more than 1.4 mm in length. Lateral hairs were brown, septate measured more than 335 µm in length and 2-4 µm in width, with few spiral coils. Ascospores were brown, aseptate, sub-globose to globose and smooth scaled 10-11 µm in length 9-10 µm in width (Fig. 1 A and B). The results of current study showed that the morphometric and microscopic characteristics of *C. bostrychodes* mentioned above almost correspond with a general pattern of fungal structures published in several related previous studies (Asgari and Zare, 2011; Abdel-Azeem, 2020; Al-Dossary *et al*., 2021). Phenotypically, the shape and size of ascospores and ascomata, and type of lateral and terminal hairs or ascomat hairs were used to classify most *Chaetomium* species (Pornsuriya *et al*., 2008; Abdel-Azeem, 2020). *C. bostrychodes* is considered important contaminant of agricultural crops, because it has ability to produce mycotoxins leading to a reduction in the quality of crop production (Asgari and Zare, 2011; Abdel-Azeem, 2020).

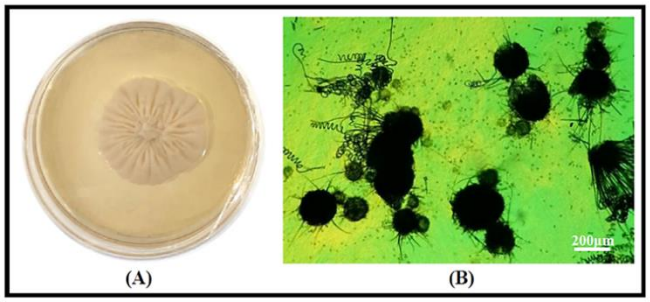

Figure 1. The morphological traits of *C. bostrychodes*. (A) Morphology of colony on PDA after 12 days, (B) Ascomata and ascospores with a magnification of 10x. Bars: B = 200 µm.

With regard to *N. novaehollandiae*, the colonies were primarily white then turn to greenish olive with dark margin within eight days. Aerial mycelia were pale to olive and septate containing serials of chlamydospores and arthroconidia. Chlamydospores were hyaline to brownish, thick wall, aseptate, cylindrical or sub-globose measured 7.5–11.5 µm in length and 2.5–3.5 µm in width. Arthroconidia were hyaline to pale brown, thick wall, cylindrical or sub-

spherical to oval measured 7.3–10.2 µm in length and 2.2–3.4 µm in width (Figure 2 A and B). The results showed that the phenotypic features of *N. novaehollandiae* mentioned above approximately resembled the forms of fungal structures reported in related previous researches (Zhu and Liu, 2012; Dervis *et al*., 2020; Oksal and Ozer 2021; Abdulrahman and Haleem, 2023). In addition to the above-mentioned, *N. novaehollandiae* is regarded as an effective pathogen causing serious plant diseases on varied plant hosts including fruit trees and agricultural crops (Abdul-Karim and Aljarah, 2021; Alizadeh *et al*. 2022). Thus, the capability of this pathogen to colonize a broad spectrum of plant hosts exhibited its pathogenicity and adaptability.

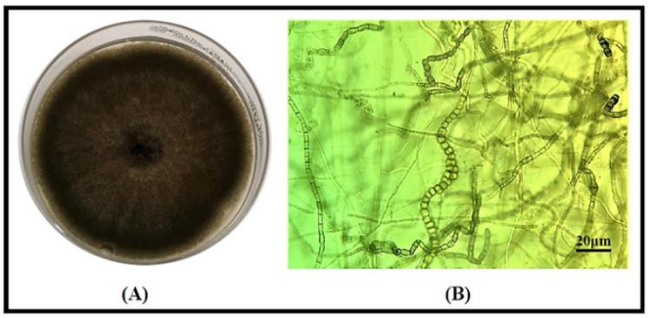

Figure 2. The morphological traits of *N. novaehollandiae*. (A) Morphology of colony on PDA after eight days, (B) Chlamydospores with a magnification of 40x. Bars: B = 20 µm.

***Molecular diagnosis*:** Regarding molecular identification of two studied fungi, the ITS1 and ITS4 were employed. The genetic sequences for both species were searched on official website of NCBI/BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi showing the exact identity of *C. bostrychodes* and *N. novaehollandiae*.

The *Chaetomium bostrychodes* was subjected to DNA analysis using the ITS primers (ITS1 and ITS4) and the PCR products of 528 bp was searched in BLASTn to confirm the identity of current fungal species and the results showed a percentage of sequence identity of 90% with the sequence accession number of *C. bostrychodes* KP216865.1 (Table 3). The Figure (3A) showed the phylogenetic tree constructed according to the maximum likelihood analysis (MEGA-11) of ITS alignments with most relevant identities, which proved that Basrah isolate of *C. bostrychodes* and the deposited species of KP216865.1 was clustered in one clade. The identity of *C. bostrychodes* has been confirmed using the ITS universal primers in many published studies (Nakayama *et al*. 2013; Linkies *et al.* 2021).

Concerning the fungus species *N. novaehollandiae*, the fungal genome was amplified using a reaction of PCR with ITS primers; and the amplicon of 517 bp was subjected to a BLASTn search to confirm the species identity; the results showed a percentage of sequence identity reached 98% with the gene accession number of MT397136.1 (Table 3). The current sequence of *N. novaehollandiae* was applied with ITS alignments for the most relevant identities to construct a phylogenetic tree using a maximum likelihood analysis (MEGA-11); the results showed that both Basrah isolate of *N. novaehollandiae* and the MT397136.1 sequence was clustered in one group as depicted in Figure (3B). This result is in accordance with findings of Alizadeh et al. (2022) and Abdulrahman and Haleem (2023) for the efficiency of ITS primers in *N. novaehollandiae* diagnosis.

Recently, many studies have proved the important role of molecular diagnosis in determining exact species of phytopathogenic fungi on major economic plants (Abass, 2017; Yaser and Abass; 2022).

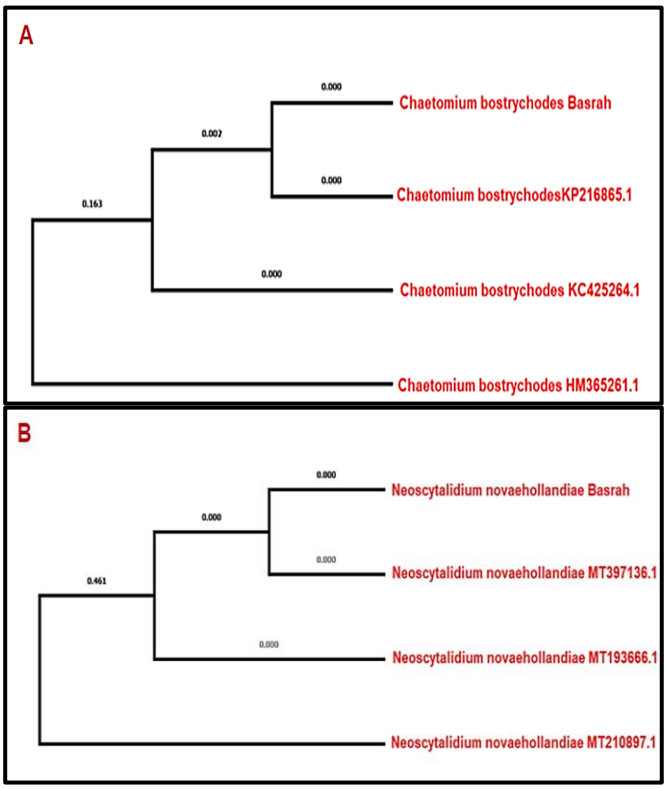

Table 3. Molecular identification of *C. bostrychodes* and *N. novaehollandiae* employing ITS primers.

Fungal species	bp length	Percentage of Sequence identity	Gen Bank accession number of organism with the highest sequence identity
C. bostrychodes	500	90	KX146494.1
N. novaehollandiae	520	98	MT397136.1

Figure 3. ***Phylogenetic trees of Chaetomium*** **bostrychodes** ***(A) and N. novaehollandiae (B).***
Maximum likelihood analysis (MLA): phylogenetic tree deduced using Internal Transcribed Spacer (ITS). The nearest three *C. bostrychodes* and *N. novaehollandiae* isolates published in GenBank (https://www.ncbi.nlm.nih.gov /genbank/samplerecord/) were used in ClustalW program in MEGA-11 to construct the tree.

Conclusion**:** The identification of two examined fungi isolated from seeds of wheat cultivars, including Mahmoudia (MHD), Babil (BBL), Bohooth (BTH), Adena (ADN) and Wefia (WAF), was based on morphological and microscopic features. This was followed by a molecular diagnosis applying ITS1 and ITS4 primers. Sequences of ITS were analyzed on website of NCBI/BLAST revealing high similarity indices of 90% with KX146494.1 and 98% with MT397136.1 for *C. bostrychodes* and *N. novaehollandiae*, respectively. It's noteworthy; that the current research is considered as the first report of both *C. bostrychodes* and *N. novaehollandiae* on wheat seeds in Iraq.

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Availability of data and material**:** We declare that the submitted manuscript is original work, which has not been published before and is not currently being considered for publication elsewhere

Authors participation**:** All authors are similarly participating in this research study for publish in JGIAS ll authors read and approved the final manuscript.

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